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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (500 characters max)

DIASTEREOMERIC PEPTIDES AS INHIBITORS OF MEMBRANE PROTEIN ASSEMBLY

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Respectfully submitted,

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PROVISIONAL PATENT APPLICATION

DIASTEREOMERIC PEPTIDES AS INHIBITORS OF MEMBRANE PROTEIN ASSEMBLY

FIELD OF THE INVENTION

The present invention relates to synthetic diastereomeric peptides corresponding to fragments of membrane proteins capable of inhibiting binding of the membrane proteins and thereby inhibiting membrane protein assembly in biological events. More particularly, the present invention relates to synthetic diastereomeric peptides, pharmaceutical compositions comprising the diastereomeric peptides, and to methods of using said compositions for inhibiting viral fusion and diseases or disorders associated with membrane protein assembly.

BACKGROUND OF THE INVENTION

Protein recognition within the membrane is crucial for a wide range of processes in all organisms. Such protein recognition is demonstrated, for example, by inter-subunit association of receptors, ion channels, transporters and pumps. Many of these biologically important membrane proteins associate via their transmembrane domains [1]. It was shown that reconstitution of a functional bacteriorhodopsin [2; 3] and Lactose permease [4] can be obtained from separate transmembrane segments. Other proteins that form hetero-oligomeric complexes are the T cell receptor [5; 6] and the MHC II complex [7]. Viral fusion proteins such as influenza hemagglutinin and Hepatitis E1/E2, and the cellular fusion protein synaptobrevin [8; 9; 10] also seem to oligomerize through their transmembrane domains. M13 major coat protein [11], phospholambdan [12], and Glycophorin A (GPA) [13] are additional membrane proteins that were shown to oligomerize via their transmembrane domains.

The assembly and oligomerization of proteins within the membrane can be categorized into three main groups based on their orientation: (i) Transmembrane orientation; (ii) Surface orientation; and (iii) Oblique orientation. All these categories may contain proteins having helical or beta sheet secondary structures or a combination of several structures.

Studies have indicated that formation of active membrane complexes can be inhibited by specific interaction of the membrane proteins with synthetic peptides. This has been demonstrated by the following examples: (i) *Transmembrane orientation* – A short mutant helical peptide from delta-endotoxin was shown to abolish the pore-forming activity of the transmembrane pore hairpin. However, other mutants having only one amino acid difference were incapable of recognizing the hairpin [14]. The assembly of a Glycophorin A peptide with the wild type transmembrane Glycophorin A *in vivo* was also demonstrated [15; 16]. The recognition process of Glycophorin A peptide within the membrane was sequence specific. Interestingly, the overall chirality of the peptide was not important for the recognition process as demonstrated by the fact that there was specific recognition within the membrane between the same Glycophorin A peptide having all D-amino acids and the wild type transmembrane domain, *in vivo* [16]. (ii) *Surface orientation* – two overlapping segments derived from HIV1 gp41 termed C34 and DP178 were previously shown to be competent inhibitors of viral fusion [17; 18; 19]. These two peptides were 100-1000 fold less active against HIV-2 strains. It was recently demonstrated that one of the major pathways through which DP178 inhibits fusion is through assembly with gp41 within the cellular membrane, arresting the fusion process in midway [20]; (iii) *Oblique orientation* – The HIV fusion peptide at the N-terminal of gp41 is known to inhibit viral fusion. The evidence collected points to a mechanism involving assembly within the membrane of the target cells [21]. Similarly to the transmembrane Glycophorin A, the fusion peptide of HIV-1 exhibits an achiral nature as the all-D fusion peptide showed similar inhibitory effect as of the all-L fusion peptide while preserving the species specificity, namely the selectivity towards HIV-2 was 100 fold lower [22].

U.S. Pat. No. 5,464,933 discloses DP178, fragments, analogs, and homologs thereof having anti-retroviral activity, specifically towards HIV. U.S. Pat. No. 5,464,933 claims DP178 as well as analogs thereof having at least one amino acid residue in a D-isomer configuration, though no specific enablement or guidance is provided for any peptide comprising a D-amino acid. U.S. Pat. No. 6,133,418 claims DP178 analogs and pharmaceutical compositions comprising thereof.

U.S. Pat. No. 6,093,794 discloses DP178, portions, and analogs thereof, which show anti-viral activity and/or anti-membrane fusion capability. U.S. Pat. No. 6,093,794 also discloses analogs of DP107, the latter is a peptide corresponding to amino acids 558-595 of the HIV_{LAI} gp41, and peptide analogs derived from an Epstein-

Barr virus protein, which were identified through computer algorithms capable of recognizing the ALLMOTI5, 107x178x4, or PLZIP amino acid motifs. Some of the peptides having structural and/or amino acid motif similarity to DP107 and DP178 are shown to exhibit activity predictive of antiviral activity. The peptides disclosed in U.S. Patent 6,093,794 may have at least one amino acid in a D-isomer configuration.

Using the same sequence searches as disclosed in U.S. Pat. 6,093,794, U.S. Pat. Nos. 6,228,983; 6,017,536; 6,013,263; and 6,020,459 disclose peptides derived from human respiratory syncytial virus, simian immunodeficiency virus, measles virus, and from HIV-1 and HIV-2, all of which are suggested to exhibit anti-fusogenic and anti-viral activities. The peptide analogs disclosed in U.S. Pat. No. 6,020,459 are suggested to have at least one amino acid in the D-isomer configuration. U.S. Pat. No. 6,518,013 discloses methods for inhibiting transmission of an Epstein-Barr virus to a cell comprising contacting the cell with a peptide derived from an Epstein-Barr virus protein, said peptide identified by the same sequence searches described above.

U.S. Pat. No. 5,840,843 provides a synthetic HIV-1 based polypeptide, which comprises an amino acid sequence corresponding to amino acid residues 600-862, preferably from 637-666 of the envelope glycoprotein of HIV-1_{IIIB}. This amino acid sequence corresponds to the fusion peptide of gp41, and it is disclosed that dimers or trimers of such peptide exhibit higher anti-fusogenic activity as compared to that obtained by the monomeric peptide.

Use of the peptides disclosed in prior art is associated with several major disadvantages. For example, as being hydrophobic, these peptides are not water-soluble. Additionally, as the amino acid residues in these peptides are primarily in an L-isomer configuration, such peptides are highly susceptible to proteolytic degradation. Also, as being short proteins they activate the immune system.

Thus, there remains an unmet need for peptides exhibiting effective inhibitory activity towards membrane protein assembly, which are water-soluble, resistant to proteolytic degradation, and having low immunogenicity. Such peptides would exhibit increased efficacy and bioavailability and would not elicit a detrimental immune response, in comparison to peptides having the native structure.

SUMMARY OF THE INVENTION

The present invention provides diastereomeric peptides capable of inhibiting binding of membrane proteins within the cell membrane and thereby inhibiting membrane protein assembly.

It is an object of the present invention to provide diastereomeric peptides capable of inhibiting binding of membrane proteins so as to inhibit membrane protein assembly. The advantages of the diastereomeric peptides are inter alia that diastereomeric peptides exhibit low immunogenicity, low susceptibility to proteolytic degradation, and water solubility.

It is another object of some aspects of the present invention to provide diastereomeric peptides having at least one of the following biological activities: anti-fusogenic activity, anti-viral activity, and/or inhibitory activity on intracellular processes involving membrane protein assembly.

It is now disclosed, for the first time, that a diastereomeric peptide comprising an amino acid sequence derived from naturally occurring membrane protein but having at least two amino acid residues in the D-isomer configuration, is capable of inhibiting membrane fusion processes.

These results were totally unexpected due to the fact that the recognition between a membrane protein and a peptide within the cell membrane was believed to be dependent upon the secondary structure of the protein and peptide. Surprisingly, diastereomeric peptides comprising an amino acid sequence derived from a membrane protein, and particularly of transmembrane segment of a membrane protein, were shown according to the present invention to inhibit membrane fusion albeit the disruption of the secondary structure of these peptide-membrane protein complexes.

The diastereomeric peptides are highly advantageous over all L- or all D-amino acid peptides having the same amino acid sequence because of their higher water solubility, lower immunogenicity, and lower susceptibility to proteolytic degradation. Such characteristics endow the diastereomeric peptides with higher efficacy and higher bioavailability compared to that of the all L or all D-amino acid peptides comprising the same amino acid sequence.

The principles of the invention are exemplified herein below by three diastereomeric peptides: DP178, a 36-mer peptide having an amino acid sequence of the

proximal region of the transmembrane domain of HIV-1_{LAVI} envelope protein gp41, a 33-mer amino terminal fusion peptide of gp41, and a 15-mer transmembrane Glycophorin A peptide. The diastereomeric peptides of gp 41 were found to inhibit cell-cell fusion events at a similar or higher efficacy than their respective all L-amino acid peptides.

The diastereomeric peptides exemplified in the present invention represent the three different categories of protein assembly and oligomerization, specifically transmembrane orientation, surface orientation, and oblique orientation. It is, therefore, explicitly intended that a wide variety of diastereomeric peptides comprising amino acid sequence of membrane proteins may be synthesized and evaluated for their activity as inhibitors of membrane protein assembly.

According to the first aspect, the present invention provides a membrane binding diastereomeric peptide comprising from about 7 to about 50 amino acid residues corresponding to an amino acid sequence of a membrane protein, the diastereomeric peptide having at least two amino acid residues in the D-isomer configuration being capable of inhibiting membrane protein assembly, an active fragment, extension, a derivative, or a salt thereof. Preferably, the diastereomeric peptide of the invention comprises from about 10 to about 40 amino acid residues corresponding to an amino acid sequence of a membrane protein.

In one embodiment, the amino acid residues of the diastereomeric peptide correspond to a transmembrane domain of a membrane protein. The membrane protein according to the invention is selected from the group consisting of viral proteins, ion channels, receptors, transporters, and pumps. In a further embodiment, the diastereomeric peptide comprises an amino acid sequence of a transmembrane domain of a viral envelope surface glycoprotein. In a preferred embodiment, the diastereomeric peptide comprises the amino acid sequence of DP178, a 36-mer peptide of the transmembrane domain of HIV-1_{LAVI} gp41. In a currently more preferred embodiment, the diastereomeric peptide is selected from diastereomeric peptides having SEQ ID NOs:1 and 2 (the D-amino acid residues are bold and underlined):

YTSLIHSLIEESQNQQEKNEQELLELDKWAS**LW**NWF (SEQ ID NO: 1)

YTSLIHSLIEE**SQ**NQQEKNEQELLELDKWASLWNWF (SEQ ID NO: 2)

The present invention also encompasses an active fragment, an extension, a derivative, or a salt of diastereomeric DP178 so long as the fragment, extension,

derivative, or salt inhibits membrane protein assembly. According to the invention, inhibitory activity towards membrane protein assembly includes at least one biological activity selected from anti-fusogenic activity, anti-viral activity, and inhibitory activity towards intracellular processes involving membrane protein assembly. It should be appreciated that the location of the D-amino acid residues in a diastereomer of the invention may vary so long as the inhibition of membrane protein assembly is maintained or enhanced.

In a further embodiment, the diastereomeric peptide of the invention comprises an amino acid sequence corresponding to gp41 amino terminal fusion peptide, a 33-mer peptide. In a currently preferred embodiment, the diastereomeric peptide is selected from diastereomeric peptides having SEQ ID NOs:3 to 5 (the D-amino acid residues are bold and underlined):

AVG**I**GAL**F**LG**F**LG**A**AGSTMGARSMTLTVQARQL (SEQ ID NO: 3)

AVGIGAL**F**LG**F**LGAAGSTMGARSMTLTVQARQL (SEQ ID NO: 4)

AVGIGAL**F**LG**F**LGAAGSTMG**A**RSMT**L**TVQARQL (SEQ ID NO: 5)

The present invention also encompasses an active fragment, an extension, a derivative, or a salt of diastereomeric gp41 amino terminal fusion peptide so long as the fragment, extension, derivative, or salt inhibits membrane protein assembly.

In another embodiment, the diastereomeric peptide comprises an amino acid sequence of a transmembrane domain of Glycophorin A. In a currently more preferred embodiment, the diastereomeric peptide is selected from diastereomeric peptides having SEQ ID NOs:6 to 9 (the D-amino acid residues are bold and underlined):

ITLIIFG**V**MAG**V**IGT (SEQ ID NO: 6)

ITLI**I**FGVMAGV**I**GT (SEQ ID NO: 7)

FSEPEITLIIFG**V**MAG**V**IGTILLISYGIRRLI (SEQ ID NO: 8)

FSEPEITLI**I**FGVMAGV**I**GTILLISYGIRRLI (SEQ ID NO: 9)

The present invention also encompasses an active fragment, an extension, a derivative, or a salt of diastereomeric Glycophorin A peptide so long as the fragment, extension, or derivative inhibits membrane protein assembly.

In another aspect, the present invention provides a pharmaceutical composition comprising as an active ingredient a membrane binding diastereomeric peptide comprising from about 7 to about 50 amino acid residues corresponding to an amino

acid sequence of a membrane protein, the diastereomeric peptide having at least two amino acid residues in the D-isomer configuration capable of inhibiting membrane protein assembly, an active fragment, extension, a derivative, or a salt thereof, and a pharmaceutically acceptable carrier.

In a further aspect, the present invention provides a method for inhibiting protein assembly in a cell comprising contacting said cell with an effective amount of a membrane binding diastereomeric peptide according to the invention, wherein said membrane protein assembly is inhibited.

In another aspect the present invention provides a method for inhibiting transmission of a virus to a cell comprising contacting said cell with an effective amount of a membrane binding diastereomeric peptide according to the invention, wherein fusion of said virus is inhibited. According to one embodiment, the method for inhibiting transmission of a virus may be utilized for inhibiting transmission of a human virus selected from HIV, human T-lymphocyte virus, leukemia virus, human respiratory syncytial virus, human parainfluenza virus, influenza virus, measles virus, and Epstein-Barr virus. However, the present invention also encompasses inhibition of viral transmission of non-human viruses selected from bovine leucosis virus, feline sarcoma virus, feline leukemia virus, simian sarcoma virus, simian leukemia virus, simian immunodeficiency virus, canine distemper virus, Newcastle disease virus, simian Mason-Pfizer virus, and sheep progress pneumonia virus.

In still a further aspect, the present invention provides a method for inhibiting virus replication or transmission in a subject comprising administering to said subject a therapeutically effective amount of a pharmaceutical composition according to the invention, whereby replication or transmission of said virus is inhibited. In one embodiment, the method for inhibiting virus replication is utilized for inhibiting replication of a virus selected from HIV, human T-lymphocyte virus, human respiratory syncytial virus, human parainfluenza virus, influenza virus, measles virus, and Epstein-Barr virus.

These and other embodiments of the present invention will be better understood in relation to the figures, description, examples, and claims that follow.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the effect of [D-LW]-DP178 on the fusion of HIV-1 gp120-41 expressing TF228 cells and CD4+ CXCR4+ 3T3 mouse fibroblasts. Solute and lipid refer to mixing of cell content and of cell membranes, respectively, by the DP178 diastereomeric peptide.

FIG. 2 shows the inhibition of virus-mediated cell-cell fusion. The effect of the diastereomeric analog of the fusion peptide of gp41 of HIV-1 (LAV1a) on the fusion of HIV-1 gp120-41 expressing TF228 cells and CD4+ CXCR4+ 3T3 mouse fibroblasts is shown (□). An 8-mer wild-type peptide derived from the N-terminal region of the fusion peptide is used as a control (◊).

FIG. 3 shows the partition of the fusion peptide and its analogs into T-cell membranes. Images show an overlay of fluorescence and transmission light. They are focused on the longitudinal section of T-cells. Panel a. WT fusion peptide. Panel b. IFFA fusion peptide. Panel c. Control cells without any peptide.

FIG. 4 shows fluorescence energy transfer dependence on the Rho-labeled peptide acceptor concentration. The spectra were obtained for a donor-peptide alone or in the presence of various amounts of an acceptor-peptide. Each spectrum was recorded in the presence of 400 μM PC:CHO SUV in PBS. The excitation wavelength was set at 467 nm; emission was scanned from 500 to 600 nm. Transfer efficiencies between donor and acceptor-WT (□), between donor-WT and acceptor-IFFA (○), and between donor-WT and acceptor-FF (Δ), are plotted versus the bound-acceptor/lipid molar ratio. A theoretical plot showing energy transfer efficiency as a function of the surface density of the acceptors, assuming random distribution of donors and acceptors, and R0=51Å, is given for comparison (line).

FIG. 5 shows the heterodimer formation through ToxR GPA inhibition. The results were normalized between wt-GPA homodimer as 0% and A_{16} monomer as 100% inhibition. All-L GPA, triangles; All-D GPA, circles; and 2D-GPA, diamonds (the previously published All-L and all-D GPA results were refitted with the refined model for comparison with 2D-GPA [15; 16]).

FIG. 6 shows the specific recognition between wt-GPA and its diastereomer *in vitro*. Theoretically and experimentally derived percentages of energy transfer efficiencies between donor wt-GPA-NBD and acceptor 2D-GPA-Rhodamine are

marked as diamonds; a negative control membrane binding monomeric NBD labeled donor and A_2-GPA-Rhodamine acceptor is marked as squares. The broken line represents a random distribution of monomers [23] using a Ro of 51Å [14].

FIG. 7 shows the expression levels of ToxR GPA in the presence and absence of 2D-GPA. Western blot analysis using anti MalE confirmed that the diastereomer exogenous peptide did not interfere with the expression of the ToxR GPA construct.

FIG. 8 shows the binding of 2D-GPA to FHK_{12} bacteria. Partitioning of NBD 2D-GPA to the inner membrane of *E. coli* was determined by confocal laser scanning microscopy. The image is focused on the longitudinal section of the bacteria. The fluorescence image is in the upper left hand; the transmission light image is in the upper right hand. An overlay of the fluorescence and transmission light is displayed in the lower panel. Most of the peptide is localized on the plasma membrane, although some peptide also penetrates into the cytoplasm. Confocal images were obtained using an Olympus IX70 FV500 confocal laser-scanning microscope. The confocal images were obtained at 12-bit resolution.

FIG. 9 presents the analysis of the structures resulting from molecular dynamics simulation. The contact surface area of single residues with the opposite helix was calculated with the CSU tool [24], the NMR model, triangles; homodimer, filled diamonds; and heterodimer, empty diamonds. Amino acids belonging to the "*interaction motif*" are marked as single letters.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides membrane binding diastereomeric peptides comprising 7 to 50 amino acid residues corresponding to a membrane protein, the diastereomeric peptides having at least two amino acid residues in a D-isomer configuration capable of inhibiting membrane protein assembly, and consequently inhibiting various biological functions associated with membrane protein assembly.

The term "diastereomeric peptide" as used herein refers to a peptide comprising both L-amino acid residues and D-amino acid residues. The number and position of D-amino acid residues in a diastereomeric peptide may be variable so long as the inhibitory activity of the peptide on membrane protein assembly is maintained or enhanced.

The diastereomeric peptides of the invention are not limited in size. However,

the invention particularly contemplates peptides having fewer than about 50 amino acid residues in total. Additionally, the peptides of the invention should include at least 7 amino acid residues, which enable the peptide to be incorporated into the lipid bilayer.

The present invention also contemplates proteins in which the core motif sequence, namely the amino acid sequences of the diastereomeric peptides of the present invention, is artificially implanted within a sequence of a polypeptide, such as peptides manufactured by recombinant DNA technology or chemical synthesis.

The diastereomeric peptides of the invention may be synthesized or prepared by techniques well known in the art. Short peptides, for example, can be synthesized on a solid support initially described by Merrifield or in solution. Longer peptides may be made using recombinant DNA techniques. Here, the nucleotide sequences encoding the peptides of the invention may be synthesized, and/or cloned, and expressed according to techniques well known to those of ordinary skill in the art. See, for example, Sambrook, et al., 1989.

The peptides of the invention may alternatively be synthesized such that one or more of the bonds, which link the amino acid residues of the peptides are non-peptide bonds. These alternative non-peptide bonds may be formed by utilizing reactions well known to those in the art, and may include, but are not limited to, imino, ester, hydrazide, semicarbazide, and azo bonds, to name but a few. The peptides of the invention may be synthesized with additional chemical groups present at their amino terminus, carboxy terminus, and/or a side chain such that, for example, the stability, bioavailability, and/or inhibitory activity of the peptides are enhanced.

It should be understood that a diastereomeric peptide of the invention need not be identical to the amino acid sequence of a naturally occurring membrane protein, so long as it includes the required sequence that allows it to be incorporated into the lipid bilayer and as such is able to inhibit the membrane protein assembly.

The term "membrane protein assembly" is used throughout the present specification to refer to complex formation between membrane proteins, which leads to membrane fusion events and/or to intracellular processes initiated by the membrane protein complex formation.

The present invention encompasses diastereomeric peptide analogs having amino acid substitutions, extensions, and/or deletions.

The amino acid substitutions may be of conserved or non-conserved nature. Conserved amino acid substitutions consist of replacing one or more amino acids of a

peptide sequence of the invention with amino acids of similar charge, size, and/or hydrophobicity characteristics, such as, for example, a glutamic acid (E) to aspartic acid (D) amino acid substitution. Non-conserved substitutions consist of replacing one or more amino acids of a peptide sequence with amino acids possessing dissimilar charge, size, and/or hydrophobicity characteristics, such as, for example, a glutamic acid (E) to valine (V) substitution. The amino acid substitutions may also include non-natural amino acids.

Amino acid extensions may consist of single amino acid residues or stretches of residues. The extensions may be made at the carboxy or amino terminal end of a peptide, as well as at a position internal to the peptide. Such extensions will generally range from 2 to 15 amino acids in length so long as the diastereomeric peptide comprises not more than 50 amino acid residues. It is contemplated that extensions made at either the carboxy or amino terminus of the peptide of interest may be of a broader size range, with about 2 to about 20 amino acids being preferred. One or more such extensions may be introduced into a peptide as long as such extensions result in peptides, which still exhibit inhibitory activity towards membrane protein assembly.

Deletions in a diastereomeric peptide are also within the scope of the invention. Such deletions consist of the removal of one or more amino acids from a naturally occurring peptide sequence, with the lower limit length of the resulting peptide sequence being 7 amino acids. Such deletions may involve a single contiguous or greater than one discrete portion of the peptide sequences. One or more such deletions may be introduced into a peptide as long as such deletions result in peptides, which still exhibit inhibitory activity towards membrane protein assembly.

The present invention also encompasses conjugates comprising the diastereomeric peptides of the invention, so long as the conjugate is capable of inhibiting complex formation of membrane proteins.

Typically, the present invention encompasses derivatives of the diastereomeric peptides. The term "derivative" includes any chemical derivative of the peptide having one or more residues chemically derivatized by reaction of side chains or functional groups. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form

O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides, which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acid residues. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted or serine; and ornithine may be substituted for lysine.

The present invention provides diastereomeric peptides comprising from 7 to 50 amino acid residues corresponding to an amino acid sequence of a membrane protein. Preferably, the diastereomeric peptides of the invention comprise from 10 to 40 amino acid residues. In a preferred embodiment, the diastereomeric peptides comprise an amino acid sequence of a transmembrane domain of a membrane protein. Typically, a transmembrane domain contains hydrophobic amino acid residues, which enable the transmembrane domain to be incorporated within the lipid bilayer.

According to the invention, the membrane binding diastereomeric peptides exhibit inhibitory activity towards membrane protein assembly. The inhibitory activity towards membrane protein assembly includes, but is not limited to, anti-fusogenic activity, anti-viral activity, and ability to modulate intracellular processes involving membrane protein assembly such as, for example, inhibitory activity of channel formation, and inhibitory activity of hormone signaling.

Assays for cell fusion events are well known to those of skill in the art. Cell fusion assays are generally performed in vitro. Such an assay may comprise culturing cells, which, in the absence of any treatment, would undergo an observable level of syncytial formation. For example, uninfected cells may be incubated in the presence of cells chronically infected with a virus that induces cell fusion. Such viruses may include, but are not limited to, HIV, SIV, or respiratory syncytial virus.

For the assay, cells are incubated in the presence of a diastereomeric peptide to be assayed. For each peptide, a range of peptide concentrations may be tested. This range should include a control culture wherein no peptide has been added.

Standard conditions for culturing cells, well known to those of ordinary skill in the art, are used. After incubation for an appropriate period (24 hours at 37°C, for example) the culture is examined microscopically for the presence of multinucleated giant cells, which are indicative of cell fusion and syncytial formation. Well-known stains, such as crystal violet stain, may be used to facilitate the visualization of syncytial

formation.

Alternatively, anti-fusogenic activity of a diastereomeric peptide of the invention may be detected by fluorescent dye transfer between labeled donor cells such as, for example, cells expressing HIV-1 gp120-41 and acceptor cells such as, for example, mouse fibroblasts, labeled with a different fluorescent dye. Addition of a peptide of the invention inhibits dye transfer, which is indicative of inhibition of cell fusion (see Example 1 herein below).

Other assay to evaluate the inhibitory activity of a diastereomeric peptide in membrane protein assembly may use the ToxR system, which is a robust method for detecting homodimerization of transmembrane domains *in vivo* (see Example 3 herein below).

Additionally, assays to test anti-viral activities of a diastereomeric peptide may be based upon measuring an enzymatic activity of a virus as a function of viral infection. If taking HIV as an example, a reverse transcriptase (RT) assay may be utilized to test a peptide ability to inhibit infection of CD-4^+ cells by cell-free HIV. Such an assay may comprise culturing an appropriate concentration (i.e., $TCID_{50}$) of virus and CD-4^+ cells in the presence of the diastereomeric peptide to be tested. Culture conditions well known to those in the art are used. A range of peptide concentrations may be used, in addition to a control culture wherein no peptide has been added. After incubation for an appropriate period (e.g., 7 days) of culturing, a cell-free supernatant is prepared, using standard procedures, and tested for the present of RT activity as a measure of successful infection. The RT activity may be tested using standard techniques such as those described by, for example, Goff et al. (Goff, S. et al., 1981, J. Virol. 38:239-248) and/or Willey et al. (Willey, R. et al., 1988, J. Virol. 62:139-147).

Standard methods, which are well known to those of skill in the art, may be utilized for assaying non-retroviral activity. See, for example, Pringle et al. (Pringle, C. R. et al., 1985, J. Medical Virology 17:377-386) for a discussion of respiratory syncytial virus and parainfluenza virus activity assay techniques.

Methods for assaying the ability of a diastereomeric peptide to modulate intracellular processes involving membrane protein assembly such as, for example, channel formation, nutrient transport, and hormone signaling are well known in the art [25; 26; 27; 28].

In vivo assays may also be utilized to test, for example, the antiviral activity of the diastereomeric peptides of the invention. To test for anti-HIV activity, for example,

the in vivo model described in Barnett et al. (Barnett, S. W. et al., 1994, Science 266:642-646) may be used.

The diastereomeric peptides of the invention may be utilized as anti-fusogenic or anti-viral compounds, or as compounds, which modulate intracellular processes involving membrane protein assembly such as, for example, channel formation, nutrient transport, and hormone signaling.

The anti-fusogenic capability of the diastereomeric peptides of the invention may additionally be utilized to inhibit or treat/ameliorate symptoms caused by processes involving membrane fusion events. Such events may include, for example, virus transmission via cell-cell fusion, abnormal neurotransmitter exchange via cell-fusion, and sperm-egg fusion. Further, the peptides of the invention may be used to inhibit free viral transmission to uninfected cells wherein such viral infection involves membrane fusion events or involves fusion of a viral structure with a cell membrane.

Retroviral viruses whose transmission may be inhibited by the diastereomeric peptides of the invention include, for example, human retroviruses, particularly HIV-1 and HIV-2, and the human T-lymphocyte viruses (HTLV-I and II). The non-human retroviruses whose transmission may be inhibited by the diastereomeric peptides of the invention include, but are not limited to, bovine leukosis virus, feline sarcoma and leukemia viruses, simian immunodeficiency virus, and sheep progress pneumonia viruses.

Non-retroviral viruses whose transmission may be inhibited by the diastereomeric peptides of the invention include, but are not limited to, human respiratory syncytial virus, human parainfluenza virus, influenza viruses, measles viruses, Epstein-Barr viruses, and hepatits B viruses. Non-human non-retroviral viruses that may be inhibited by the diastereomeric peptides of the invention include, but are not limited to, simian Mason-Pfizer viruses, canine distemper virus, and Newcastle disease virus.

Non enveloped viruses whose transmission may be inhibited by the diastereomeric peptides of the invention include, but are not limited to, picornaviruses such as polio viruses, hepatitis A virus, enterovirus, echoviruses and coxsackie viruses, papovaviruses such as papilloma virus, parvoviruses, adenoviruses and reoviruses.

The anti-viral activity of the peptides of the invention may show a pronounced type and subtype specificity, i.e., specific diastereomeric peptides may be effective in inhibiting the activity of only specific viruses. This feature of the invention presents

many advantages. One such advantage, for example, lies in the field of diagnostics, wherein one can use the antiviral specificity of the diastereomeric peptide of the invention to ascertain the identity of a viral isolate.

Among the intracellular disorders involving membrane protein assembly, which may be ameliorated by the diastereomeric peptides of the invention are disorders involving, for example, bacterial toxins.

In another aspect, the present invention provides a pharmaceutical composition comprising a therapeutically effective amount of a membrane binding diastereomeric peptide of the invention having an inhibitory activity on membrane protein assembly and a pharmaceutically acceptable carrier.

A pharmaceutical composition useful in the practice of the present invention typically contains a diastereomeric peptide of the invention formulated into the pharmaceutical composition as a pharmaceutically acceptable salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide), which are formed with inorganic acids, such as for example, hydrochloric or phosphoric acid, or with organic acids such as acetic, oxalic, tartaric, and the like.

Suitable bases capable of forming salts with the diastereomeric peptides of the present invention include, but are not limited to, inorganic bases such as sodium hydroxide, ammonium hydroxide, potassium hydroxide and the like; and organic bases such as mono-, di- and tri-alkyl and aryl amines (e.g. triethylamine, diisopropyl amine, methyl amine, dimethyl amine and the like) and optionally substituted ethanolamines (e.g. ethanolamine, diethanolamine and the like).

A therapeutically effective amount of a peptide of the invention is an amount that when administered to a patient is capable of exerting an inhibitory activity on membrane protein assembly and hence on membrane fusion events such as, for example, viral transmission, and intracellular processes involving protein membrane assembly. Preferably, a pharmaceutical composition is useful for inhibiting a viral disease in a patient as described further herein. In this embodiment, a therapeutically effective amount is an amount that when administered to a patient is sufficient to inhibit, preferably to eradicate, a viral disease.

The preparation of pharmaceutical compositions, which contain peptides as active ingredients, is well known in the art. Typically, such compositions are prepared as injectable, either as liquid solutions or suspensions, however, solid forms, which can

be suspended or solubilized prior to injection, can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is mixed with inorganic and/or organic carriers, which are pharmaceutically acceptable and compatible with the active ingredient. Carriers are pharmaceutically acceptable excipients (vehicles) comprising more or less inert substances when added to a pharmaceutical composition to confer suitable consistency or form to the composition. Suitable carriers are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, stabilizers, and anti-oxidants, which enhance the effectiveness of the active ingredient.

The pharmaceutical composition can be delivered by a variety of means including intravenous, intramuscularly, infusion, oral, intranasal, intraperitoneal, subcutaneous, rectal, topical, or into other regions, such as into synovial fluids. However delivery of the composition transdermally is also contemplated, such by diffusion via a transdermal patch.

In another aspect the present invention provides a method for inhibiting membrane protein assembly in a cell comprising contacting said cell with an effective amount of a membrane binding diastereomeric peptide according to the invention, wherein said membrane protein assembly is inhibited.

In a further aspect, the present invention provides a method for inhibiting transmission of a virus to a cell comprising contacting said cell with an effective amount of a membrane binding diastereomeric peptide according to the invention, wherein fusion of said virus is inhibited.

In still a further aspect, the present invention provides a method for inhibiting virus replication and transmission in a subject comprising administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising a diastereomeric peptide of the invention dispersed in a pharmaceutically acceptable carrier.

Patients in which the inhibition of viral replication would be clinically useful include patients suffering from diseases transmitted by various viruses including, but are not limited to, diseases transmitted by HIV-1 and HIV-2, human respiratory syncytial virus, human parainfluenza virus, measle virus, Epstein-Barr virus, hepatitis B virus, polio virus, and a like. It should be appreciated that Epstein-Barr virus is a human herpes virus, which is the causative agent of, for example, infectious mononucleosis

(MN), and is also associated with nasopharyngeal carcinomas (NPC), Burkitt's lymphoma, and other diseases. Additionally, disorders involving bacterial toxins may also be treated/ameliorated by the pharmaceutical composition comprising a diastereomeric peptide of the invention.

The composition is administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, and the capacity of the subject's blood hemostatic system to utilize the active ingredient. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual.

Methods of treating a disease according to the invention may include administration of the pharmaceutical compositions of the present invention as a single active agent, or in combination with additional methods of treatment. The methods of treatment of the invention may be in parallel to, prior to, or following additional methods of treatment.

EXAMPLE 1

DP178 Diastereomer

Peptides Synthesis

DP178 diastereomeric analogs were synthesized by using the Boc chemistry described by Merrifield et al. [29]. Peptides were cleaved from the resins by HF, precipitated with ether and then purified by reverse-phase HPLC on an analytical C18 Vydac column 4.6 mm X 250 mm (pore size of 300 Å). The peptides were eluted by a linear gradient of 25-80% acetonitrile in water containing 0.05% TFA (v/v), at a flow rate of 0.6 ml/min for 80 minutes. Concentrations were measured by tryptophan and tyrosine absorbance (at 280 nm) in 8M Urea [30].

Dye transfer fusion assay

Peptide inhibition of cell-cell fusion was assayed by monitoring the redistribution of two fluorescent probes, water soluble probe and lipophilic probe, between target and effector cells upon their co-incubation with these probes [31]. The HIV-1 gp120-41 expressing TF228 cells [32] were labeled with either calcein or a green fluorescent fatty acid as follows: HIV-1 gp120-41 expressing TF228 cells were incubated with 1 μM

calcein for 60 minutes at 37°C, and then washed and resuspended at 10^5 cells/ml in RPMI medium. Alternatively, the HIV-1 gp120-41 expressing TF228 cells were labeled with the fatty acid (4,4-difluoro-5-methyl-4-bora-3a, 4a-diaza-s-indacene-3-dodecanoic acid (C1-BODIPY-C12)) as follows: HIV-1 gp120-41 expressing TF228 cells were seeded at 10^5 cells/ml in RPMI medium containing 10 mg/ml of the C1-BODIPY-C12 (Molecular Probes, Eugene, OR) and grown for three days prior to the experiment [33]. The fatty acid was eventually metabolized into phospholipid, primarily phosphatidylcholine. The cells where then washed and resuspended at 10^5 cells/ml. The target cells, CD4+ CXCR4+ 3T3 mouse fibroblasts, were plated at 10^5 cells/ml the night before the experiment. The next day, the CD4+ CXCR4+ 3T3 cells were labeled with 20 μM [5-(and-6) ((4-Chloromethyl)benzoyl)amino)) tetramethylrhodamine (CMTMR) for one hour at 37°C, washed several times and combined with the effector cells (1:3 target-effector cell ratio). Different concentrations of peptides, dissolved in PBS or Tris buffer, were then added. The cells were co-cultured for 2 hours at 37°C in 12 well plates (Costar, Cambridge, MA). Phase and fluorescent images were collected using an Olympus IX70 coupled to a CCD camera (Princeton instruments, Trenton, NJ) with a 20x objective lens. An 82000 optical filter cube (Chroma technology corp., Brattleboro, VT) was used for the excitation of calcein (494/517), BODIPY (500/510), and CMTMR (541/565). Three images per well were collected and then analyzed using metamorph software (Universal imaging, West Chester, PA) for dye transfer from the donor to the acceptor cell. The scoring of fusion events was conducted as described by Munoz-Barroso et al. [31].

Results

Inhibition of cell-cell fusion induced by DP178 diastereomer

There are two possible targets for inhibition by DP178 [18]: (i) DP178 inhibits the formation of the heterotrimeric coiled-coil gp41 by interacting with the leucine/isoleucine zipper sequence in the aqueous solution via its N-terminal region; (ii) fusion pore formation is inhibited by the interaction of C-terminal part of DP178 with another target site within the membrane.

In an attempt to correlate between the structure and the inhibitory activity of DP178, two diastereomeric peptides were synthesized:

(i) A diastereomer of DP178 having two adjacent D-amino acid residues at the N-terminal region of DP178, D-S^{649}, Q^{650}-DP178 (designated herein D-SQ) having the following amino acid sequence (the D-amino acid residues are bold and underlined):

YTSLIHSLIEE**SQ**NQQEKNEQELLELDKWASLWNWF (SEQ ID NO: 2)

Using structurally constrained analogs, McDowell and coworkers demonstrated that the overlapping part of C34 (overlapping with the N-terminal region of DP178) and DP178 inhibits infectivity by adopting a helical conformation [34]. This occurs when the inhibitor interacts with the coiled-coil forming sequence in aqueous solution. It was therefore expected that D-S^{649}, Q^{650}-DP178 will not be able to inhibit HIV-1 induced membrane fusion.

(ii) A diastereomer of DP178 having two adjacent D-amino acid residues at the C-terminal region of DP178, D-L^{669}, W^{670}-DP178 (herein designated D-LW) having the following amino acid sequence:

YTSLIHSLIEESQNQQEKNEQELLELDKWAS**LW**NWF (SEQ ID NO: 1)

It was postulated that if this diastereomer is an inhibitor for HIV-1 infection, it would indicate that the dominant target site of the C-terminal region of DP178 is within the membrane.

FIG.1 shows the effect of [D-LW]-DP178 on dye transfer fusion assay between HIV-1 gp120-41 expressing TF228 cells and CD4+ CXCR4+ 3T3 mouse fibroblasts. As shown in FIG. 1, [D-LW]-DP178 inhibited the fusion of TF228 cells with 3T3 fibroblasts. Under similar experimental conditions, [D-SQ]-DP178 was completely inactive. Remarkably, [D-LW]-DP178 was more active than the wild-type DP178.

EXAMPLE 2

HIV-1 Fusion Peptide Diastereomer

We have synthesized a diastereomeric peptide, representing the N-terminal 33 amino acid segment of gp41 of HIV-1 (LAV1a) in which four D-amino acid residues IFFA were incorporated. The peptide has the following amino acid sequence:

AVG**I**GAL**F**LG**F**LG**A**AGSTMGARSMTLTVQARQL (SEQ ID No: 3)

Results

Inhibition of cell-cell fusion induced by the wt-fusion Peptide and its diastereomeric analog.

The diastereomeric and the wild type peptides were tested for inhibitory effect on HIV-1 mediated cell-fusion as described herein above (Example 1). The diastereomer exhibited significant inhibitory effect similarly to the wild type peptide already at a concentration of 10 ng/ml (FIG. 2). An 8-mer and a 13-mer N-terminal regions of the fusion peptide as well as the fusion peptide of Sendai virus were used as controls for the specificity of HIV-1 inhibition. These control peptides were all completely devoid of inhibitory effect, suggesting that the inhibition of the WT and its diastereomeric analog is specific for HIV-1.

Binding of wt-fusion peptide and its diastereomeric analog to T-cells.

Nitrobenzofurazane (NBD)-labeled wt-fusion peptide or NBD-labeled fusion peptide diastereomer (herein designated IFFA) were studied for their binding to activated T-cells as follows: Activated T-cells (10,000) were incubated for 30 minutes at room temperature with NBD labeled WT or IFFA Fusion peptides (0.5 mM final concentration). The cells were washed twice with PBS (100 ml) in order to remove excess unbound peptide. The cells were then observed under the fluorescent confocal microscope. NBD excitation was at 488 nm with the laser set at 2% power to prevent bleaching of the fluorophore. Fluorescence data was collected from 525 nm and up. Localization of the peptides to the T-cells was monitored by confocal fluorescence microscopy and is shown in FIG. 3. As shown in FIG. 3, both the wt-fusion peptide and IFFA demonstrated high affinity binding towards T-cells. The peptides localized at the plasma membrane and formed patches of high intensity (FIG. 3), suggesting that both peptides have preferential binding to lipid rafts on the membrane of T-cells. When sliding across the z-axis it becomes obvious that the peptides do not penetrate the cytoplasmic membrane and no peptide was observed within any other membrane compartment in the cells.

Coassembly of wt-fusion peptide and its diastereomeric analog in the membrane-bound State.

The self-association of the peptides in their membrane-bound state was monitored by employing resonance energy transfer measurements. Fluorescence resonance energy

transfer was measured using NBD (nitrobenzofurazane)-labeled peptides serving as donors and Rho (rhodamine)-labeled peptides serving as energy acceptors [35]. Fluorescence spectra were obtained at room temperature with excitation set at 467 nm using a 10 nm slit width. In a typical experiment, donor peptide (final concentration 0.04 μM) was added to a dispersion of phosphatidylcholine/cholesterol (PC/Chol; 10:1 w/w) small unilamellar vesicles (SUV; 224 μM) in PBS, followed by the addition of acceptor peptide in several sequential doses. Fluorescence spectra were obtained before and after addition of the acceptor. The efficiency of energy transfer (E) was determined by measuring the decrease in the quantum yield of the donor as a result of the presence of acceptor. E was determined experimentally from the ratio of the fluorescence intensities of the donor in the presence (I_{da}) and in the absence (I_d) of the acceptor, at the donor's maximum emission wavelength. The percentage of transfer efficiency (E), is given by:

$$E = (1 - I_{da} / I_d) \times 100\%$$

Correction for the contribution of acceptor emission as a result of direct excitation was made by subtracting the signal produced by the acceptor-labeled analog alone. The contribution of buffer and vesicles was subtracted from all measurements. A dose dependent quenching of the NBD-wt-fusion peptide donor's emission, consistent with energy transfer, was observed when Rho-wt-fusion peptide or Rho-IFFA were added (final concentration of 0.025 μM to 0.35 μM) to a mixture of NBD-WT (0.05 μM) and SUV (400 μM). The energy transfer was calculated and plotted as a function of the molar ratio of peptide acceptor/lipid (FIG. 4). The acceptor-peptide was added only after the donor-peptide was already bound to the membrane, thus decreasing association in solution. The lipid/peptide molar ratio in these experiments was kept high in order to create low surface density of donors and acceptors to reduce energy transfer between unassociated peptide monomers. In order to confirm that the observed energy transfer is due to peptide aggregation, the transfer efficiencies observed in the experiments were compared with the energy transfer expected for randomly distributed membrane-bound donors and acceptors (FIG. 4). The random distribution was calculated as described by Fung and Stryer [23], assuming that 51 Å is the R_0 value for the NBD/Rho donor/acceptor pair [36]. The levels of energy transfer between the different pairs are significantly higher then those expected for random distribution of donors and acceptors. The results clearly demonstrate an ability of the IFFA to associate with the wt-fusion peptide.

EXAMPLE 3

Glycophorin-A Diastereomer

Glycophorin A (GPA) has a known homodimerization motif, the core of which is GxxxG. Previous results demonstrated that the GPA transmembrane can dimerize in an achiral manner, while keeping wild type like structure and interactions [16]. In order to understand the role of the helical secondary structure in the assembly process, we synthesized a diastereomer analog (herein designated 2D-GPA) of the GPA transmembrane. Two L-valines at positions 80 and 84 according to the 1AFO pdb structure, each following a glycine in the GxxxG motif, were replaced by their D-enantiomer. The amino acid sequence of the diastereomeric peptide of GPA is as follows:

ITLIIFGV̲MAGV̲IGT (SEQ ID NO: 6)

Glycines are known to be a weak point for helices and together with the replacement of the large β-branched valines by their D-enantiomer, they are bound to form a large disturbance in the secondary structure [37; 38; 39]. The ability of the diastereomeric peptide to interact with the wild type GPA transmembrane in the membrane of bacteria was tested using the ToxR system [40]. In addition, a molecular dynamic simulation was used to try and assess the effect of the structural disturbance on the heterodimer structure formed.

The diastereomer 2D-GPA hetero-associates with the all-L GPA transmembrane

The ToxR GPA plasmid construct is a simple and robust method for detecting homodimerization of transmembrane domains *in vivo* [40]. The detection is done through the signal of a β-Galactosidase reporter gene. Heterodimerization can be detected through addition of an exogenous peptide. Interaction of this peptide with a ToxR GPA receptor within the membrane will form a heterodimer with an inactive ToxR transcription factor. The 2D-GPA peptide was exogenously added to the system in a dose dependent manner (FIG. 5). The concentration ranged from 0.5-20 μM. A concentration dependent dominant negative effect was observed. Inhibition was calculated as follows: I is the inhibitory ability of the peptide, $A_{peptide}$ is the activity of ToxR-GPA in the presence of peptide, A_{max} is the maximal activity of ToxR-GPA without peptide, and $A_{baseline}$ is the baseline activity of the monomer A_{16} plasmid [40].

(1) $$I = 1 - \frac{A_{peptide} - A_{baseline}}{A_{max} - A_{baseline}}$$

IC_{50} was calculated by fitting normalized data to the Langmuir equation (equation 3) in the Origin 6.0 commercial program [41].

(2) $$AA + B \Leftrightarrow A + AB$$

(3) $$I = 1 - \frac{k * IC_{50}}{[B] + IC_{50}}$$

Were A represents ToxR-GPA transcription factor, B represents the exogenous peptide, I represents inhibition of beta-galactosidase activity (1-activity) and K is a scaling constant.

The IC_{50} of the interaction between 2D-GPA and the integral GPA transmembrane domain, as extrapolated from the fit, was 0.29 μM with R^2 of 0.95. When fitting previous all L and all D GPA results to this model an IC_{50} of 0.36μM and 0.48μM were extrapolated with R^2 of 0.92 and 0.95, respectively.

In vitro recognition between WT-GPA and the diastereomer peptide

Wild type-GPA and the 2D-GPA analog were synthetically labeled with Rhodamine and NBD, respectively and fluorescent resonance energy transfer (FRET) experiments were performed with NBD-labeled peptides serving as energy donors and Rhodamine-labeled peptides serving as energy acceptors. Fluorescence spectra were obtained at room temperature, with excitation set at 467 nm (8 nm slit) and emission measured at 520 nm (16 nm slits). In a typical experiment, a donor peptide (final concentration 0.1 μM) was added to a dispersion of LUV (340 μM) in PBS. This was followed by the addition of an acceptor peptide in several sequential doses ranging from 0.01 to 0.05 μM. At this peptide to lipid ratios complete membrane binding is expected for all the transmembrane peptides. Fluorescence spectra were obtained before and after addition of the acceptor. The efficiency of energy transfer (E) was determined by measuring the decrease in the quantum yield of the donor as a result of the presence of the acceptor. E was determined experimentally from the ratio of the fluorescence intensities of the donor in the presence (I_{da}) and in the absence (I_d) of the acceptor, at the

wavelengths of the maximal donor's emission. The percentage of transfer efficiency (E), is given by:

(4) $$E\ (\%) = (1 - I_{da} / I_d) \times 100$$

Subtracting the signal produced by the acceptor-labeled analogue alone made correction for the contribution of acceptor emission as a result of direct excitation. The contribution of buffer and vesicles was subtracted from all measurements. The R_0 for this pair of donor-acceptor fluorophores is around 51Å. The dose dependent FRET is shown in FIG. 6. Different pairs of membrane binding peptides that do not assemble within the membrane were tested as a control and were found to have a much lower energy transfer, as expected.

ToxR receptor expression levels

FHK12 cells were grown in the presence or absence of 2D-GPA, 10μl samples were added to 2x sample buffer and boiled for 5 minutes. The samples were separated on a 12% SDS-PAGE and thereafter were blotted. The primary antibody used was anti Maltose binding protein. The detection was performed with "Phototope-HRP Western Blot Detection System" (Cell Signaling Technology).

The expression levels of the ToxR GPA construct in FHK_{12} bacteria was compared in the presence or absence of the 2D-GPA exogenous peptide. As shown in FIG. 7, no significant differences were detected in the presence of 2D-GPA, thus excluding the possibility that the peptide modifies the expression levels of the ToxR GPA construct. These results are in agreement with the previously reported results for the all L- and all D-GPA peptides [16]. Therefore, the differences in ToxR signal as observed herein above are a consequence of direct interaction of the receptor with the exogenous peptide within the bacterial membrane.

Binding of 2D-GPA to the FHK12 bacterial membrane

The binding of 2D-GPA to the membrane of the FHK12 bacteria is a prerequisite for the interaction between the peptide and the GPA ToxR construct. In order to verify this binding, the bacteria were observed under a confocal microscope in the absence or presence of a NBD labeled 2D-GPA peptide at a 500 nM concentration. Experiments were done as follows: FHK_{12} bacteria from the control of the association assay were diluted 1:10000 and incubated with NBD labeled 2D-GPA, final concentration of 500 nM, for 30 seconds. Samples were fixed with Gluteraldehyde at a final concentration of

0.001% for 30 seconds, centrifuged and washed 3 times with fresh PBS. Confocal images were obtained using an Olympus IX70 FV500 confocal laser-scanning microscope. Care was taken that any photobleaching did not compromise the interpretation, and laser irradiation and other illumination was prevented between acquisitions. The confocal images were obtained at 12-bit resolution.

As shown in FIG. 8, the 2D-GPA peptide is localized in the bacterial membrane. This observation is consistent with previously published data for the all L- and all D-GPA peptides. Thus, the present results indicate that the diastereomer 2D-GPA can bind and insert into the bacterial membrane inspite of the structural disruption caused by the substitution of the two beta-branched L-valines with their D-enantiomer.

Molecular Dynamic Simulation

Molecular dynamics provides a putative structural rationalization to the observed phenomenon. More specifically, the NMR structure of the all-L homodimer was utilized to produce a heterodimer composed of an all-L and a diastereomer chain [28]. Both homodimer and heterodimer were subjected to a molecular dynamics procedure in vacuum to simulate the membrane environment [16; 42; 43]. The resulting homodimer and heterodimer structures were compared and analyzed for resemblance (FIG. 9).

A close look into individual atom contacts of amino acids within the *"interaction motif"* reveals a high similarity. Leu75 and Ile76 form strong inter-chain hydrophobic interactions. Gly79 and Gly83 from chain A have a CA backbone interaction with Val80 and Val84 from chain B. Gly83 from chain A interacts with Gly83 from chain B and the latter interacts also with both Val80 and Val84 from chain B. These interactions confirm that the GxxxG motif, which forms the basis of the knob-into-grove stacking of the two GPA helices, is present in the heterodimer in spite of the two D-Valines. Another strong interaction that drives the dimerization of the helices is the polar-polar interaction between the side chain Thr87 from the two chains.

Overall comparison of the inter-helical contact surface with that of the *"interaction motif"* suggests a major role for the motif in both homodimer and heterodimer structures. The total surface of the inter-helix contact in the resulting structures was 1264±156Å^2 for the homodimer and 1105±156Å^2 for the heterodimer. The surface area of the inter-helix contacts for residues forming the *"interaction motif"* was 782±72Å^2 for the homodimer and 702±23Å^2 for the heterodimer, representing

62±12 and 64±14 percent out of the total contact surface, respectively. Furthermore, when comparing the contact surface of each residue in the dimer with the opposite helix, it becomes clear that the *"interaction motif"* is playing an important role in the dimerization of both the homodimer and heterodimer (FIG. 9).

These results indicate that disrupting the secondary structure of the GPA peptide with D-amino acids does not alter significantly the dimerization propensity of the transmembrane GPA. The present findings also imply that "wild type" like dimer structure can be formed within the membrane albeit the large disruption of the secondary structure of one helix. Thus, the membrane is presumably involved in the remarkable preservation of wild type like strucuture.

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described herein above. Rather the scope of the invention is defined by the claims that follow.

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CLAIMS:

1. A membrane binding diastereomeric peptide comprising from 7 to 50 amino acid residues corresponding to an amino acid sequence of a membrane protein, the diastereomeric peptide having at least two amino acid residues in the D-isomer configuration capable of inhibiting membrane protein assembly, an active fragment, an extension, a derivative, or a salt thereof.

2. The diastereomeric peptide according to claim 1 comprising from 10 to 40 amino acid residues..

3. The diastereomeric peptide according to claim 1 wherein the amino acid residues correspond to a transmembrane domain of a membrane protein.

4. The diastereomeric peptide according to claim 3 wherein the membrane protein is selected from viral proteins, ion channels, receptors, transporters, and pumps.

5. The diastereomeric peptide according to claim 4 wherein the viral protein is a viral envelope surface glycoprotein.

6. The diastereomeric peptide according to claim 5 wherein the viral envelope surface glycoprotein is selected from HIV, human T-lymphocyte virus, human respiratory syncytial virus, human parainfluenza virus, influenza virus, measles virus, and Epstein-Barr virus.

7. The diastereomeric peptide according to claim 6 wherein the viral envelope surface glycoprotein is HIV-1_{LAVI} gp41.

8. The diastereomeric peptide according to claim 7 is a diastereomer of DP178 selected from SEQ ID NO:1 and 2.

9. The diastereomeric peptide according to claim 7 is a diastereomer of gp41 amino terminal fusion peptide selected from SEQ ID NO:3 to 5.

10. The diastereomeric peptide according to claim 4 wherein the membrane protein is Glycophorin A.

11. The diastereomeric peptide according to claim 10 is selected from SEQ ID NO: 6 and 7.

12. A pharmaceutical composition comprising as an active ingredient a membrane binding diastereomeric peptide comprising from 7 to 50 amino acid residues corresponding to an amino acid sequence of a membrane protein, the diastereomeric peptide having at least two amino acid residues in the D-isomer configuration capable of inhibiting membrane protein assembly, an active fragment, extension, a derivative, or a salt thereof, and a pharmaceutically acceptable carrier.

13. The pharmaceutical composition according to claim 12 wherein the diastereomeric peptide comprises from 10 to 40 amino acid residues.

14. The pharmaceutical composition according to claim 12 wherein the amino acid residues of the diastereomeric peptide correspond to a transmembrane domain of a membrane protein.

15. The pharmaceutical composition according to claim 14 wherein the membrane protein is selected from viral proteins, ion channels, receptors, transporters, and pumps.

16. The pharmaceutical composition according to claim 15 wherein the viral protein is a viral envelope surface glycoprotein.

17. The pharmaceutical composition according to claim 16 wherein the viral envelope surface glycoprotein is selected from HIV, human T-lymphocyte virus, human respiratory syncytial virus, human parainfluenza virus, influenza virus, measles virus, and Epstein-Barr virus.

18. The pharmaceutical according to claim 17 wherein the viral envelope surface glycoprotein is HIV-1_{LAVI} gp41.

19. The pharmaceutical composition according to claim 18 wherein the diastereomeric peptide is a diastereomer of DP178 selected from SEQ ID NO:1 and 2.

20. The pharmaceutical composition according to claim 18 wherein the diastereomeric peptide is a diastereomer of gp41 amino terminal fusion peptide selected from SEQ ID NO:3 to 5.

21. The pharmaceutical composition according to claim 15 wherein the membrane protein is Glycophorin A.

22. The pharmaceutical composition according to claim 21 wherein the diastereomeric peptide is selected from SEQ ID NO:6 and 7.

23. A method for inhibiting membrane protein assembly in a cell comprising contacting said cell with an effective amount of a membrane binding diastereomeric peptide according to any one of claims 1 to 11, wherein said membrane protein assembly is inhibited.

24. A method for inhibiting transmission of a virus to a cell comprising contacting said cell with an effective amount of a membrane binding diastereomeric peptide according to any one of claims 1 to 11, wherein fusion of the virus is inhibited.

25. The method according to claim 24 wherein the virus is selected from HIV, human T-lymphocyte virus, human respiratory syncytial virus, human parainfluenza virus, influenza virus, measles virus, and Epstein-Barr virus.

26. A method for inhibiting virus replication or transmission in a subject comprising administering to said subject a therapeutically effective amount of a pharmaceutical composition according to any one of claims 12 to 22, whereby replication or transmission of said virus is inhibited.

27. The method according to claim 26 wherein the virus is selected from HIV, human T-lymphocyte virus, human respiratory syncytial virus, human parainfluenza virus, influenza virus, measles virus, Epstein-Barr virus, and Hepatitis B virus.

28. A method for treating a disease or disorder associated with an intracellular process involving membrane protein assembly comprising administering to said subject a therapeutically effective amount of a pharmaceutical composition according to any one of claims 12 to 22, whereby the intracellular processes involving assembly of membrane proteins are inhibited.

ABSTRACT

The present invention relates to membrane binding diastereomeric peptides comprising amino acid sequences corresponding to transmembrane domains of membrane proteins, said peptides having at least two amino acid residues in a D-isomer configuration capable of inhibiting membrane protein assembly. The diastereomeric peptides are useful in inhibiting fusion membrane protein events, specifically viral replication and transmission.

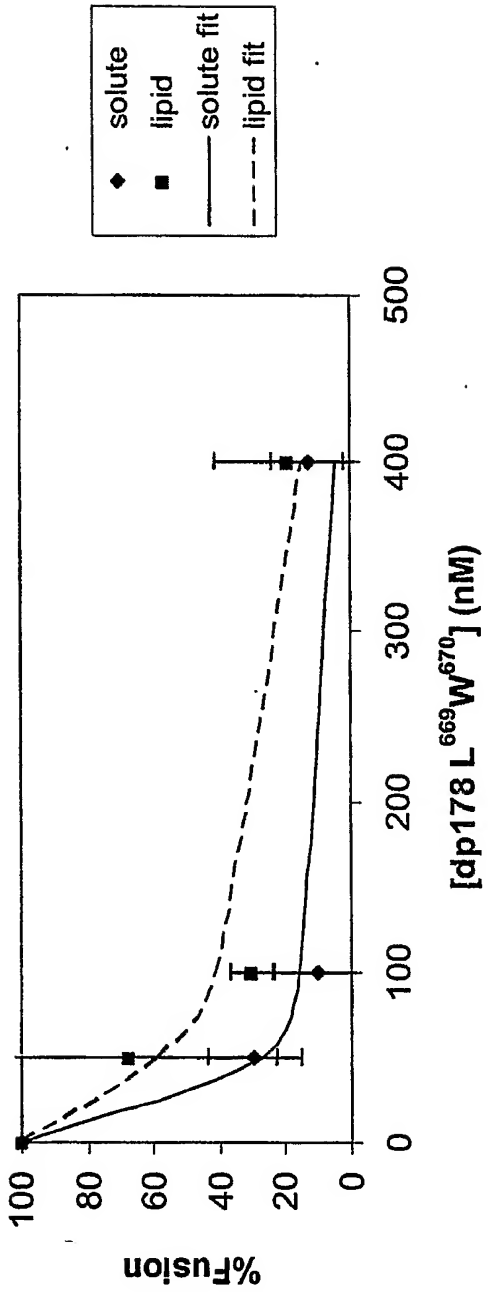

FIG. 1

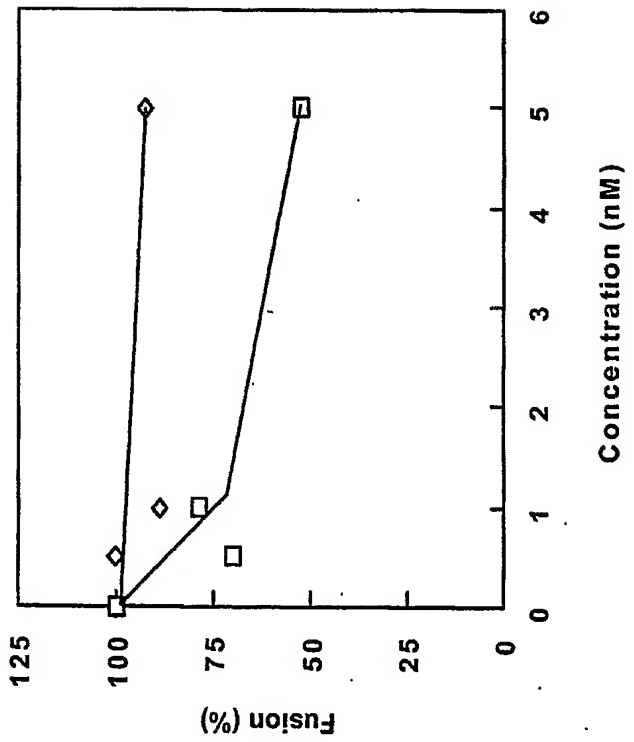

FIG. 2

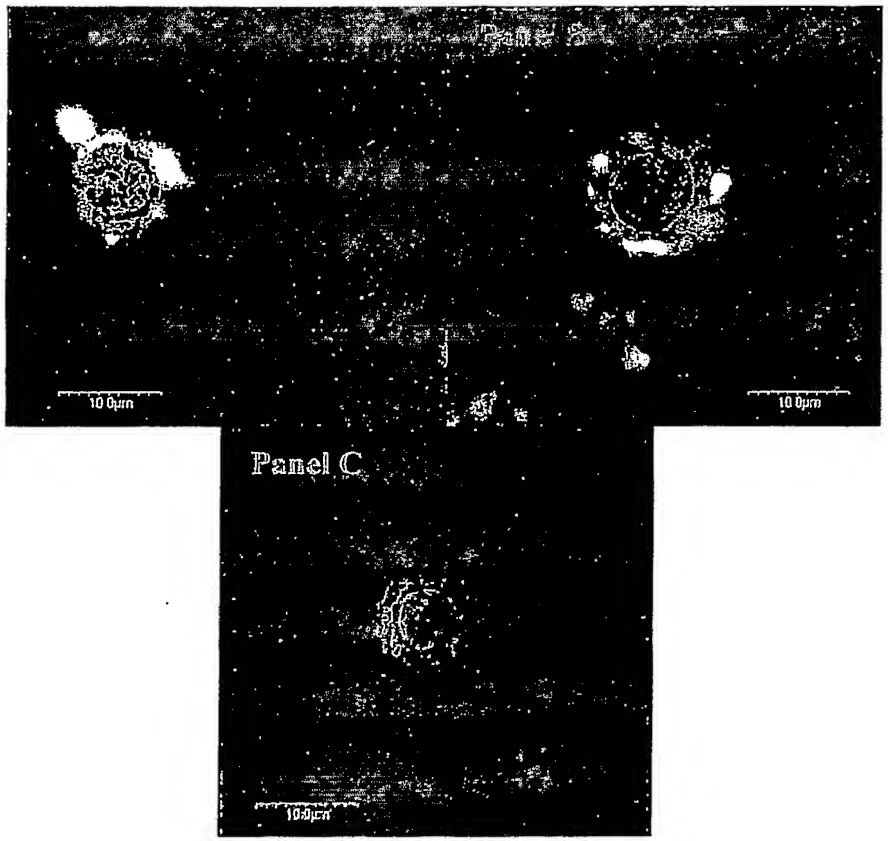

FIG. 3A-C

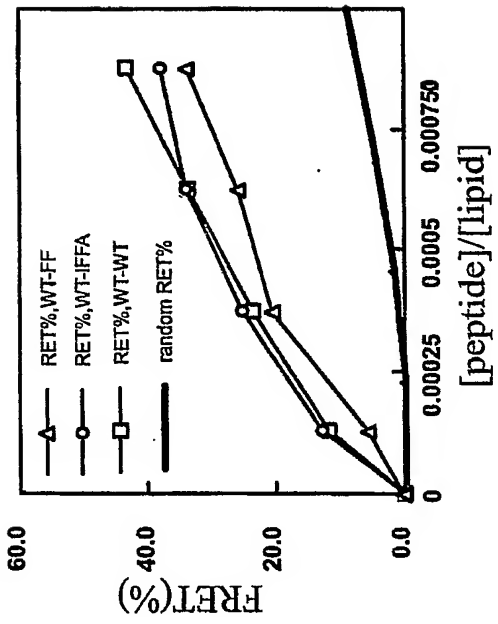

FIG. 4

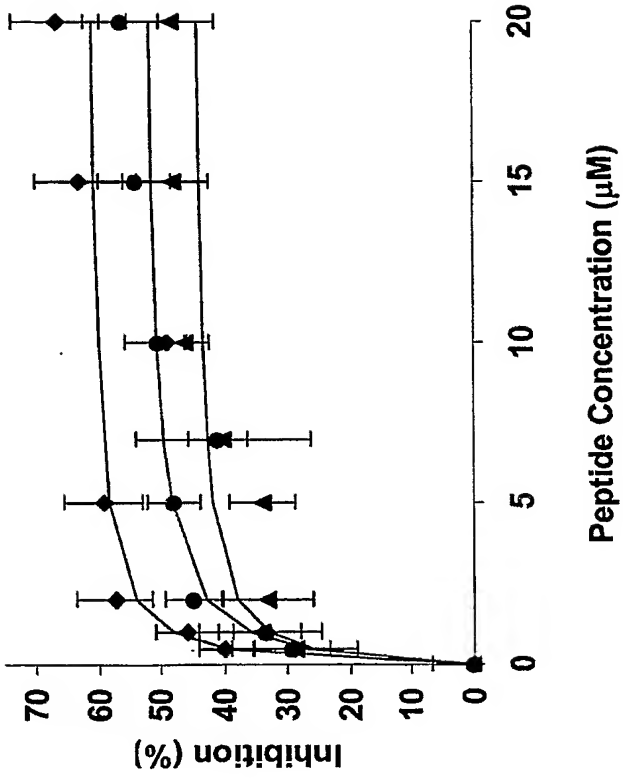

FIG. 5

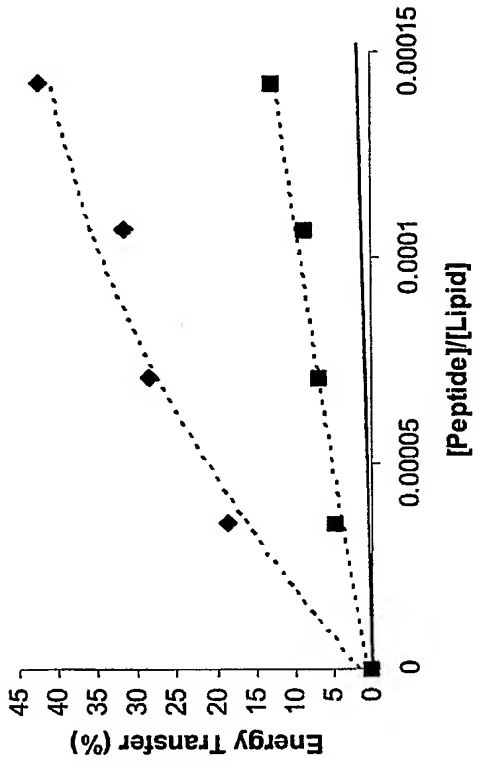

FIG. 6

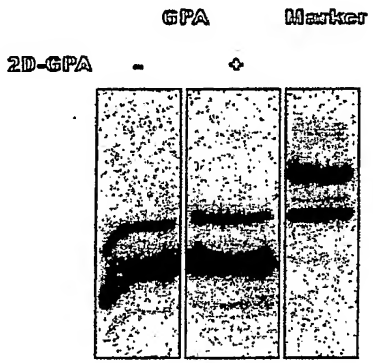

FIG. 7

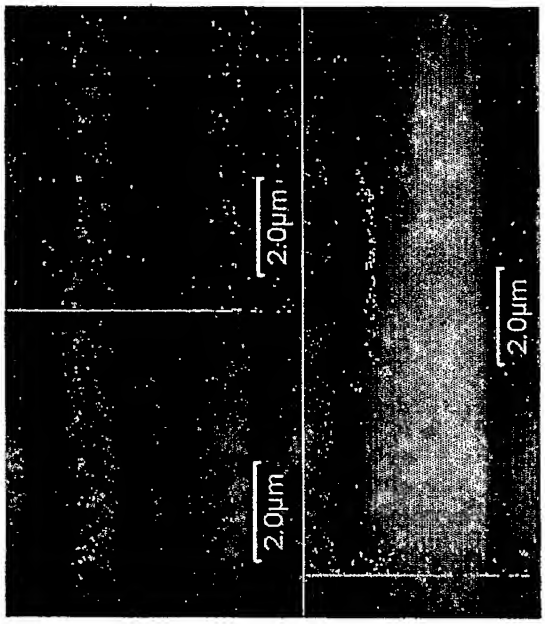

FIG. 8

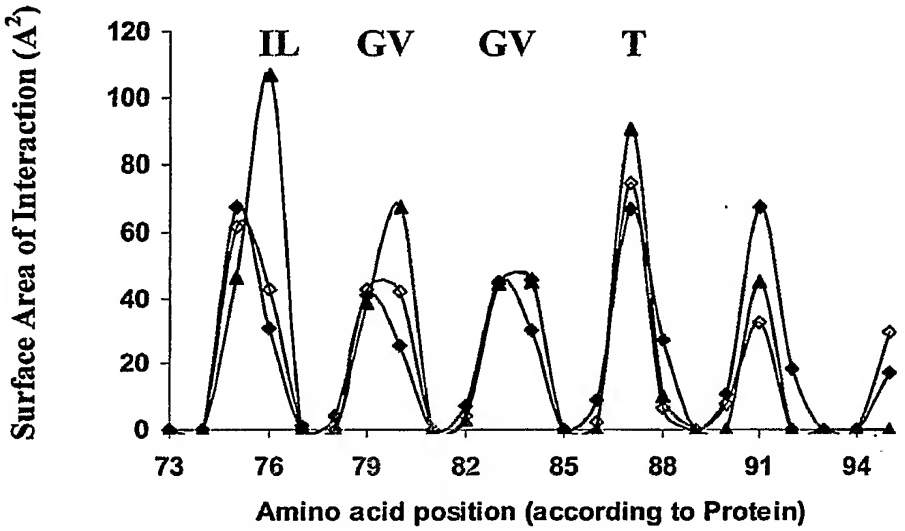

FIG. 9